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Effect of 2 Emulsion-Based Adjuvants on the Structure and Thermal Stability of *Staphylococcus aureus* Alpha-Toxin

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ABSTRACT

The effects of 2 squalene-based emulsion adjuvant systems (MedImmune emulsion 0 [ME.0] and Stable Emulsion [SE]) on the structure and stability of the recombinant protein antigen alpha-toxin (AT), a potential vaccine candidate for *Staphylococcus aureus* infection, were investigated using Fourier-transform infrared spectroscopy and both steady-state and time-resolved intrinsic fluorescence spectroscopy as well as differential scanning calorimetry (DSC). A component study, performed to identify the effects of the individual emulsion's components, showed negligible interactions between AT and ME.0. DSC analysis showed the ME.0 emulsion thermally destabilized AT, probably because of changes in the buffer composition of AT upon mixing. The SE emulsion caused increased alpha-helix and decreased beta-sheet content in AT, and a significant blue shift in the fluorescence spectra relative to that of AT in solution. DSC analysis showed SE exerted a dramatic thermal stabilization effect on AT, probably attributable to an interaction between AT and SE. Size exclusion chromatography showed a complete loss in the recovery of AT when mixed with SE, but not ME.0, indicating a high degree of interaction with SE. This work successfully characterized the biophysical properties of AT in the presence of 2 emulsion adjuvants including a component study to rationalize how emulsion components affect protein antigen stability.

Introduction

Subunit vaccines contain defined macromolecular components identified from a pathogen that are capable of eliciting protective immunity.[1] They are usually recombinant proteins and possess many advantages over other vaccine types (e.g., live attenuated and inactivated viruses and bacteria) such as an improved safety profile, a highly defined nature, ease of production, and potential for lower cost of goods.[1,2] One important limitation of subunit vaccines is that they usually induce relatively weak immunogenicity owing to their inability to replicate and lack of other immunostimulatory components such as pathogen-associated molecular pattern molecules to induce innate and cellular immunity.[3] To ensure the successful immunization of subunit vaccines, they are often administered with adjuvants to boost host immune responses.[3]

Oil-in-water (O/W) emulsion-based adjuvants are an important class of adjuvants used for subunit vaccines.[4-6] These emulsions usually use squalene as the oil phase, which is a naturally occurring lipid found in plants, animals, and humans. Suitable surfactants (e.g., Tween 80, Span 85 etc.) are used to stabilize oil droplets dispersed in the aqueous environment. Currently, 2 squalene-based emulsion adjuvants (MF59 and AS03) have been approved for commercial use.[7-9] Potent immunopotentiators, such as monophosphoryl lipid A, can be added to emulsion-based adjuvants to further improve their adjuvanticity. For example, a squalene emulsion containing a glucopyranosyl lipid adjuvant, a synthetic form of monophosphoryl lipid A, is currently used in a phase II trial for a respiratory syncytial virus vaccine.[10,11] In this work, MedImmune emulsion 0 (ME.0) and Stable Emulsion (SE) O/W emulsions were investigated. Both contain squalene as the oil phase. ME.0 and SE use histidine and ammonium phosphate buffers as their aqueous phase, respectively. The surfactants used in ME.0 and SE are PS 80 and a combination of 1, 2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and Pluronic

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F68, respectively. In ME.0, sucrose is employed as an osmolyte, whereas glycerol is used in SE. SE also contains vitamin E, which serves as an antioxidant and has also been shown to exert immunostimulatory activity.[12,13]

The protein antigen studied in this work is alpha-toxin (AT) derived from *Staphylococcus aureus*. AT is one of the key virulent factors involved in *S aureus* infection[14] and has been found to be protective in animal models.[15] AT is a promising vaccine candidate for protection against antibiotic resistant staphylococcus infection in high-risk populations, especially patients hospitalized for surgery.[7] The wild type AT is expressed by *S aureus* extracellularly as a water soluble monomer and self-assembles into a homo-oligomeric heptamer with a transmembrane domain.[16] The AT monomer consists of 293 amino acid with a molecular weight of 33.2 kDa. The AT used in this study is a mutant of the wild type AT, which lacks the ability to self-assemble.

Emulsion adjuvants can be formulated with vaccine antigens in 2 different formats: single vial or separate vials in which antigen and adjuvant are mixed prior to administration.[17] If antigen and adjuvant are compatible with each other, they can be formulated in a single vial to reduce manufacturing costs and provide ease of administration. Single vial formulation is therefore a more favorable option, especially as the commercial dosage form.[2] However, if antigens are not compatible with adjuvants over long-term storage, they must be stored separately before administration to ensure the safety and efficacy of a vaccine. This option is often implemented in early clinical trials when limited stability data are available. An understanding of emulsion/antigen interactions and how these interactions influence the structure and stability of antigens is necessary to rationally decide the type of formulation (e.g., single vial or separate vial) and to further optimize the formulation to offer maximum antigen stability.[18-20]

Since emulsions are usually optically turbid due to strong light scattering, this poses challenges for analytical characterization of antigens in their presence. Many routinely used spectroscopic techniques (such as circular dichroism [CD], UV absorption, and some forms of fluorescence) are often not suitable for this purpose because of the interference by light scattering.[21] In the present study, a set of biophysical techniques capable of analyzing turbid protein samples were selected to investigate the secondary, tertiary structural integrity, and overall conformation stability of protein antigens upon mixing with emulsion-based adjuvants. These techniques include Fourier-transform infrared spectroscopy (FTIR) and intrinsic fluorescence spectroscopy and differential scanning calorimetry (DSC).[22] We have successfully used these techniques to characterize the effects of 2 O/W emulsion systems (designated as ME.0 and SE) on *S aureus* AT, a potential vaccine antigen.

Materials and Methods

Materials

Frozen stock of recombinantly produced and purified AT in phosphate-buffered saline (PBS) (pH 7.4) was obtained from Medimmune LLC (Gaithersburg, MD). The AT stock concentration was found to be 0.51 mg/mL (extinction coefficient, $A_{\frac{0.1\%}{280\ nm}} = 1.93$) by UV absorption spectroscopy, and this value was used for sample preparation. Two O/W emulsion-based adjuvants, ME.0 stock (2X) and SE stock (3X), were provided by MedImmune LLC. ME.0 stock (2X) contains 20-mM histidine at pH 6.0, 10% sucrose, 4% squalene, and 1% PS80. SE stock (3X) is composed of 25 mM ammonium phosphate, 5.1% squalene, 1.1% DMPC, 30 μg/mL vitamin E, 2.37% w/v glycerol, and 0.037% w/v pluronic F68, at pH 5.6.

Sample Preparation

ME.0 stock (2X) and SE stock (3X) were prepared by MedImmune LLC using a previously described protocol.[2] Briefly, squalene and surfactant were mixed and sonicated to achieve homogenous mixing of the oil phase. The aqueous phase was then added to the oil phase. The mixture was again homogenized using a Silverson L5M-A standard mixer (East Longmeadow, MA) and then subjected to microfluidization to generate milky emulsions of around 100 nm in size using a Microfluidics 110P microfluidizer (Microfluidics, Boston, MA). AT/ME.0 or AT/SE mixtures were prepared by mixing AT in PBS with an emulsion stock (ME.0 stock [2X] or SE stock [3X]) to achieve a final protein concentration of 0.2 mg/mL containing emulsion at 1X concentration.

In the component study, we aimed to study the effects of different emulsion components on AT (as per Table 1). AT was mixed with each water-soluble component of the emulsions at the same volumetric ratio as used in the AT/ME.0 or AT/SE mixture. S0 was prepared by diluting AT in PBS to achieve a concentration of 0.2 mg/mL. The compositions of all test samples and their identification codes (M1 to M5 and S1 to S5) are listed in Table 1. For example, M1 was prepared by mixing AT in PBS (at 0.4 mg/mL) with 20-mM histidine buffer, pH 6.0 at a volumetric ratio of 1:1. M2 was made by mixing AT in PBS (at 0.4 mg/mL) with a mixture solution containing 20-mM histidine buffer (pH 6.0) and 10% sucrose at a volumetric ratio of 1:1. ME.0 (1X) and SE (1X) diluted using PBS were used as control samples. The mixtures were equilibrated at 4°C overnight before analysis.

UV-Visible Absorption Spectroscopy

The absorption spectrum of AT stock was collected using a UV/visible spectrophotometer (Agilent Technologies, Santa Clara, CA) equipped with a diode array detector. The absorption spectra were corrected for scattering contributions by subtraction of an extrapolation of the logarithm of the optical density in a nonabsorbing region (350 to 400 nm) to the far UV region.

FTIR Spectroscopy

FTIR spectroscopic analysis was performed using a Tensor-27 FTIR spectrometer (Bruker, Billerica, MA) equipped with a BioATR cell. The detector was cooled with liquid N_2 for 20 min prior to use, and the interferometer was purged continuously with N_2 gas. A total of 256 scans were recorded from 4000 to 900 cm^{-1} with a 4 cm^{-1} resolution. Buffer/emulsion background spectra were collected and subtracted from the sample spectra. Atmospheric compensation, baseline adjustment, and second derivative calculations were applied using OPUS V6.5 (Bruker, Billerica, MA) software. To compare the initial state of the samples, spectra collected at 20°C were deconvoluted into a set of mixed Gaussian/Lorentzian bands, using the build-in Levenberg-Maquardt algorithm from the OPUS V6.5 software. Thermal unfolding experiments were performed with the temperature ramped from 20°C to 90°C or 99°C (for AT in SE) at increments of 2.5°C per step and an equilibration time of 2 min at each temperature. The second derivative of each spectrum was calculated with 9 point smoothing. The thermal unfolding curves of AT were constructed by plotting the second derivative signal at 1621 cm^{-1} (an indication of intermolecular β-structure and protein aggregation) as a function of temperature. The melting temperature (T_m) was calculated by a first derivative method using Origin 2017 (OriginLab, Northampton, MA).